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(21) International Application Number: PCT/US98/03636 (22) International Filing Date: 24 February 1998 (24.02.98) (30) Priority Data: 60/038,797 24 February 1997 (24.02.97) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/038,797 (CIP) Filed on 24 February 1997 (24.02.97) (71) Applicant (for all designated States except US): TM TECHNOLOGIES, INC. [US/US]; 82 Cummings Park, Woburn, MA 01801 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): LANE, Michael, J. [US/US]; 3044 Claybourne Lane, Baldwinsville, NY 13027 (US). BENIGHT, Albert, S. [US/US]; 1630 Valley View Drive, Schaumburg, IL 60193 (US). FALDASZ, Brian, D. [US/US]; 4 Bellevue Terrace, Maynard, MA 01754 (US). MACADAM, Don [CA/CA]; 318 Grace Street, Port Dover, Ontario N0A 1N0 (CA).		(74) Agents: DECONTI, Giulio, A., Jr. et al.; Lahive & Cockfield, LLP, 28 State Street, Boston, MA 02109 (US). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: ANTI-SENSE OLIGONUCLEOTIDE PHARMACOLOGICAL AGENTS (57) Abstract Anti-sense oligonucleotide pharmacological agents, and methods of making and using them, are disclosed.		

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ANTI-SENSE OLIGONUCLEOTIDE PHARMACOLOGICAL AGENTS

Background of the Invention

Antisense therapy involves the administration of exogenous oligonucleotides that
5 bind to a target nucleic acid, typically an RNA molecule, located within cells. The term
antisense is so given because the oligonucleotides are typically complementary to
mRNA molecules ("sense strands") which encode a cellular product. The ability to use
anti-sense oligonucleotides to inhibit expression of mRNAs, and thereby to inhibit
protein expression *in vivo*, is well documented. However, selection of an appropriate
10 complimentary oligonucleotide (or oligonucleotides) to a given mRNA is not always
simple (see, e.g., Crooke, S.T. *FASEB J.* 7: 533-539 (1993), incorporated herein by
reference). Anti-sense agents typically need to continuously bind all target RNA
molecules so as to inactivate them or alternatively provide a substrate for endogenous
ribonuclease H (Rnase H) activity. Sensitivity of RNA/oligonucleotide complexes,
15 generated by the methods of the present invention, to Rnase H digestion can be
evaluated by standard methods (see, e.g., Donia, B. P., et al., *J. Biol. Chem.* 268
(19):14514-14522 (1993); Kawasaki, A. M., et al., *J. Med. Chem.* 6(7):831-841 (1993),
incorporated herein by reference). Anti-sense oligonucleotides which are as long as the
entire mRNA compliment have been used to modulate mRNA translation, but have
20 disadvantages associated with large sequences, e.g., pharmacological availability.
Furthermore, long oligonucleotides may have less ability to discriminate between target
sequences which differ in only one (or a few positions).

It is believed that shorter (15-200) base anti-sense molecules would be preferred
in certain clinical applications. Shorter anti-sense oligonucleotides may be more
25 bioavailable or more easily formulated, and can generally be synthesized by chemical
methods more easily than can longer sequences. However, shorter oligonucleotides
must be selected to have sufficient stability to hybridize to the target nucleic acid *in vivo*,
and must also have sufficient length to bind selectively to the target sequence, while
avoiding excessive binding to non-target sequences.

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Summary of the Invention

The present invention relates to anti-sense therapy, e.g., for disease management
or eradication. The invention further relates to methods for selecting tandem
oligonucleotides for use as anti-sense agents *in vivo* or *in vitro*.

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In one aspect, the invention provides a method of treating a condition related to
expression of mRNA, comprising administering to a subject in need thereof an effective
amount of a first anti-sense oligonucleotide complementary to a first region of a target

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mRNA sequence and a second anti-sense oligonucleotide complementary to a second mRNA sequence, the first and second anti-sense regions of the target mRNA sequence being substantially contiguous, such that the condition is treated. In a preferred embodiment, the target mRNA sequence is a mutant sequence, i.e., a sequence which differs from a normal or wild-type nucleic acid sequence by one or more nucleotide bases. In a preferred embodiment, the first and second anti-sense oligonucleotides are selected such that the first and second anti-sense oligonucleotides can hybridize to the target (e.g., mutant) sequence while not significantly hybridizing to the target (e.g., native or wild-type) sequence, such that selective therapy is provided.

In another aspect, the invention provides a method for providing tandem anti-sense oligonucleotides for use in anti-sense therapy. The method includes the steps of selecting first and second regions of a target nucleic acid sequence (e.g., of a subject organism), the first and second regions being substantially contiguous; and preparing first and second anti-sense oligonucleotides, the first anti-sense oligonucleotide being complementary to the first region of the target nucleic acid sequence, and the second anti-sense oligonucleotide being complementary to the second region of the target nucleic acid sequence, such that tandem anti-sense oligonucleotides are provided. In preferred embodiments, at least one of the first and second antisense oligonucleotides is perfectly complementary to a respective region of a mutant nucleic acid sequence of an organism, but is not perfectly complementary (e.g., at one or more base positions) to a normal or wild-type nucleic acid sequence of an organism.

In another aspect, the invention provides a method for discriminating between two different target RNA molecules whose sequences differ by a single base.

In yet another aspect, the invention could be used to preferentially inhibit those cells containing a single base variant of a virus while allowing cells without the single base change virus variant to not be inhibited.

In another aspect, the invention provides kits comprising tandem anti-sense oligonucleotides in a container, preferably with instructions for using the tandem anti-sense oligonucleotides in a therapeutic method.

In another aspect, the invention provides tandem anti-sense oligonucleotides in a pharmaceutically acceptable carrier.

In another aspect, the invention provides a method of treating a subject suffering from a disorder, where the disorder is characterized by the presence of a mutation in at least one portion of gene, comprising administering to the subject in need thereof, an effective amount of first and second oligonucleotides which bind to substantially contiguous sites of the mutant form of the gene while substantially not binding to the normal form of the gene.

In another aspect, the invention provides a method of treating a subject with a disorder, wherein the disorder is characterized by the presence of a mutation in a nucleic acid sequence of the subject (e.g., an mRNA sequence of the subject), comprising administering to a subject in need thereof, an effective amount of at least two
5 oligonucleotides which bind cooperatively to the mutant gene, such that the subject is treated. The at least two oligonucleotides, which preferably are capable of hybridizing to substantially contiguous portions of the nucleic acid sequence of the subject, can optionally be administered in a pharmaceutically acceptable carrier.

In a further aspect, the invention provides a method of treating a subject having a
10 proliferative disorder characterized by abnormal gene expression, comprising administering to the subject in need thereof, an effective amount of the first and second antisense oligonucleotides such that the treatment occurs.

In another aspect, the invention provides a method of treating a viral infection in a subject, comprising administering to a subject in need thereof, an effective amount of
15 first and second antisense oligonucleotides, such that the first and second antisense oligonucleotides contact a target viral nucleic acid, such that a duplex is formed, and preventing expression of a protein encoded by the target viral nucleic acid, such that treatment of the viral infection occurs.

20 *Brief Description of the Drawings*

Figure 1 schematically depicts the hybridization of tandem anti-sense primers to target and non-target sequences.

Figure 2 depicts melting curves of matched and mismatched sequences to a
25 target "hairpin" sequence.

Detailed Description of the Invention

The invention relates to anti-sense therapies, to methods for selecting anti-sense oligonucleotides for use in anti-sense therapies, and tandem anti-sense nucleotides useful
30 in anti-sense therapies.

As used herein, the term "antisense" nucleotides, oligonucleotides or nucleic acid are interchangeable and refer to a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, e.g., complementary to the coding strand of a double-stranded cDNA molecule, complementary to an mRNA sequence or
35 complementary to the coding strand of a gene.

In one aspect, the invention provides a method of treating a condition related to expression of mRNA. The method comprises administering to a subject in need thereof

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an effective amount of a first anti-sense oligonucleotide complementary to a first region of a target mRNA sequence and a second anti-sense oligonucleotide complementary to a second region of the target mRNA sequence, the first and second anti-sense regions of the target mRNA sequence being substantially contiguous. such that the condition is
5 treated.

In another embodiment, the invention provides a method for providing tandem anti-sense oligonucleotides for use in anti-sense therapy. The method includes the steps of selecting first and second regions of a target nucleic acid sequence, the first and second regions being substantially contiguous; and preparing first and second anti-sense
10 oligonucleotides, the first anti-sense oligonucleotide being complementary to the first region of the target nucleic acid sequence, and the second anti-sense oligonucleotide being complementary to the second region of the target nucleic acid sequence, such that tandem anti-sense oligonucleotides are provided.

The term "tandem oligonucleotides" as used herein, refers to oligonucleotides
15 which are complementary to substantially contiguous, non-overlapping sequences of target nucleic acid. Tandem oligonucleotides can include first and second antisense oligonucleotides (also referred to herein as "probes"); in certain embodiments, a third tandem oligonucleotide (tandem to either the first or the second antisense probe), a fourth tandem oligonucleotide, or higher numbers of oligonucleotides, can be used.

The term "substantially contiguous", as used herein, refers to regions or portions
20 of a nucleic acid which are directly adjacent to each other or are separated by no more than one intervening nucleotide base. Thus, tandem oligonucleotides of the present invention which bind to substantially contiguous portions of a target nucleic acid will hybridize directly adjacent to each other or separated by a gap no more than one base
25 wide. Tandem oligonucleotides can bind cooperatively to the target nucleic acid (see, e.g. Kandlmall et al. *Nucleic Acids Research* (1995) 23:3578-3584). Thus, in preferred embodiments, tandem antisense oligonucleotides are selected such that binding of a first antisense oligonucleotide to a complementary target sequence can promote the binding of a second, tandem antisense oligonucleotide to the target sequence.

In preferred embodiments, the first and second anti-sense oligonucleotides are
30 selected such that the anti-sense oligonucleotides selectively bind to a target sequence which differs from non-target sequences (which are preferably not significantly bound by the antisense probes) by a single base. Such sequence specificity addresses an important issue in current anti-sense therapeutic designs. For example, use of selective
35 tandem anti-sense oligonucleotides permits discrimination between cells which include a native gene or mRNA and cells which include a mutant gene or mRNA, where the mutant differs from the native sequence by a single base. Thus, for example, a

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cancerous cell, in which one a single base of a gene has mutated, can be selectively targeted by appropriate choice of tandem anti-sense oligonucleotides, e.g., choice of tandem oligonucleotides which are perfectly complementary to the mutant sequence, but differ from, and do not bind significantly to, or bind less well to, the native sequence.

- 5 Thus, the mutant cell can be targeted without significantly affecting the normal, non-target cells. Accordingly, the invention provides methods for selectively affecting a pre-selected target cell while not significantly affecting a non-target cell.

In a preferred embodiment, the first anti-sense oligonucleotide is 5-50 nucleotides in length, more preferably 10-25 nucleotides in length, and the second anti-sense oligonucleotide is 5-50 nucleotides in length, more preferably 10-25 nucleotides in length, so that when they are combined the total length of tandem, anti-sense oligonucleotides bound to the target nucleic acid totals 10-105 nucleotide bases, more preferably 15-50, and most preferably 20-30 nucleotide bases.

- 15 In another embodiment, the ratio of the length of the first (e.g., shorter) anti-sense oligonucleotide to the length of the second (e.g., longer) anti-sense oligonucleotide is at least 1:1, 1:2, 1:3, or 1:4.

In another embodiment, the ratio of the length of the first anti-sense oligonucleotide to the length of the second anti-sense oligonucleotide is at least 2:1, 3:1, or 4:1.

- 20 In a preferred embodiment, the invention provides a method of treating a subject suffering from a disorder which is characterized by the presence of a mutation in a nucleic acid sequence of the subject, comprising administering to the subject in need thereof, an effective amount of at least two oligonucleotides preferentially binding to the site of the mutant nucleic acid sequence (e.g., gene or mRNA) such that the subject is treated. In such preferential binding, the anti-sense oligonucleotides are substantially contiguous. The term "preferential binding" as used herein, refers to the selective binding of the antisense oligonucleotides to the mutant gene rather than the wild type gene.

- 30 The invention further contemplates a method of treating diseases caused by mutations in tumor suppresser genes, e.g., P53. Mutations in proto-oncogenes can also be treated according to the methods of the invention. Certain types of mutant proto-oncogenes are known to those skilled in the art and these include, for example group c-myc, c-myb, c-fos, ras and BCR/AB.

- 35 In a preferred embodiment, the invention provides a method of treating a human subject with a disorder said disorder characterized by the presence of a mutation in a nucleic acid sequence of the subject, comprising administering to the subject in need thereof in a pharmaceutically acceptable carrier, an effective amount of at least two

oligonucleotides forming a duplex at the site of the mutant nucleic acid sequence (e.g., gene or mRNA), such that the subject is treated. Such tandem anti-sense oligonucleotides when forming a duplex are preferably substantially contiguous and are able to discriminate between the wild-type gene and the mutant gene of the subject.

5 In a particularly preferred embodiment, the invention provides a method of treating a human subject having a proliferative disorder which is characterized by abnormal gene expression, by administration of an effective amount of the first and second antisense oligonucleotides. Such proliferative disorders are characterized by uncontrolled proliferation of epithelial cells gut-derived epithelial cells, for example, in
10 some colorectal cancers.

In another particularly preferred embodiment, the invention provides a method of treating a viral infection in a human subject, by administration of an effective amount of the first and second antisense oligonucleotides, binding the viral nucleic acid, forming a duplex and preventing gene expression in the virus, such that treatment occurs.

15 Examples of viral targets include papillomavirus, herpes simplex virus and HIV-1.

Another example of a suitable target condition is sickle cell anemia. Sickle cell anemia is known to be caused by a single nucleic acid mutation, which results in a single amino acid substitution in hemoglobin. Persons who are heterozygous for the sickle cell trait possess one normal and one mutant gene. Therefore, treatment according to the
20 method of the present invention, e.g., using tandem antisense oligonucleotides which are perfectly complementary to the mutant form of the gene, but which are not perfectly complementary to the normal form of the gene) can provide selective suppression of the mutant form.

Other examples of disorders characterized by the presence of a point mutation in
25 a gene of a subject include hemophilia and Charcot-Marie-Tooth disease. Other diseases or conditions which can be treated according to the methods of the invention will be apparent to one of ordinary skill in the art.

The tandem anti-sense oligonucleotides employed can preferentially "stack" onto the single base mutant RNA (the energetic difference provides for a specificity of, e.g.,
30 10-100 fold). The largest energy difference between binding to a mutant (e.g., a point mutant) sequence and a native sequence will generally occur when the tandem oligonucleotides hybridize to contiguous regions of the target sequence, and the single-base change occurs at the "nick" formed by the hybridization of the tandem oligonucleotides, i.e., when a terminal base at the "nick" of one of the tandem
35 oligonucleotides is mismatched relative to the wild-type sequence. In contrast, when the same pair of oligomers encounter the wild-type sequence, efficient hybridization is inhibited by virtue of the mismatched base pair at the nick. The first and second anti-

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sense oligonucleotides are preferably selected such that the stabilization resulting from base stacking at the nick results in efficient hybridization to the pre-selected target sequence, but efficient hybridization does not occur to sequences which differ from the target sequence (for a description of base stacking energetics, see, e.g., Broude et al. (Proc. Natl. Acad. Sci. USA 91:3072 (1994) (incorporated by reference) and references cited therein). Such a result can be achieved by selecting at least one of the tandem anti-sense oligonucleotides such that at least one of the tandem oligonucleotides does not stably hybridize to the target sequence in the absence of the other tandem anti-sense oligonucleotide (e.g., the energy of base stacking at the nick is sufficient to promote efficient hybridization when both of the tandem oligonucleotides are present and hybridized to the target). The energetics of hybridization are dependent at least in part upon the length and base composition of the target and the anti-sense probes, as will be apparent to one of ordinary skill in the art. Accordingly, the skilled artisan will be able to select appropriate tandem anti-sense probes for a selected target sequence in light of the teachings provided herein.

The selectivity advantage described above can be provided by designing a pair of oligonucleotides that will anneal in a tandem fashion forming a "nick" precisely at the position of a point mutation. The principle is illustrated in Figure 1. In the top panel of Figure 1 ("Case 1"), two tandem oligonucleotides ("A-S Oligonucleotide 1" and "A-S Oligonucleotide 2" are shown hybridized to two contiguous regions of a target sequence ("Target", in this case a target sequence which includes a point mutant compared to the native form). The two anti-sense probes are perfectly complementary to the target and form a duplex with a "nick" (the two probes are not covalently joined).

In the lower panel of Figure 1, ("Case 2"), the two anti-sense oligonucleotides are not perfectly complementary to the target (in this case a native or wild-type target sequence) and a single base pair mismatch results at the site of the single base point mutation. As a result, hybridization in this case is less efficient than in Case 1. Thus, selectivity for the target sequence over the mutant sequence is achieved.

It will be appreciated from the above discussion that two tandem anti-sense oligonucleotides chosen to be complementary to a mutant sequence (as in Case 1) can selective bind to a mutant target sequence, while not significantly binding to a native target sequence which differs from the mutant sequence by a single base. Accordingly, the mutant target can be selectively targeted by anti-sense therapy.

The greater selectivity of the perfectly annealed anti-sense oligonucleotide pair, based on an inherent stability gain at a perfect "nick", is graphically demonstrated in Figure 2. In Figure 2, four melting profiles (which can be obtained by conventional methods, see, e.g., U.S. Patent No. 5,593,834 to Lane *et al.*, incorporated herein by

reference) are shown for the reaction of a nucleic acid "hairpin" (shown at the top of Figure 2) with both a "perfect" (i.e., perfectly complementary) 13-mer and three "imperfect" (e.g., not perfectly complementary) 13-mers. The melting curves shown in Figures 2A-D show that for certain "imperfectly stacked" (notably an A-C and a T-C (cases C and D, Figures 2C and 2D, respectively)) an advantage is gained of ~5°C in melting temperature for the mismatched anti-sense oligonucleotides compared to the perfectly complementary tandem oligonucleotides (Case A, Figure 2A). The difference in melting temperature corresponds to lower stability of the mismatched oligonucleotides when the mismatched base is located at a nick between two tandem anti-sense oligonucleotides. The differential selectivity advantage of a two oligonucleotide anti-sense strategy provides the ability to discriminate point mutants from native sequences.

Exemplary (but not limiting) potential target sequences are proto-oncogenes, for example, including but not limited to the following: c-myc, c-myb, c-fos, c-kit, ras, and BCR/ABL (e.g., Wickstrom, E., Editor, "Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS", Wiley-Liss, New York, N.Y. (1991); Zalewski, A., et al., *Circulation Res.* 88:1190-1195 (1993); Calabretta, B., et al., *Seminars in Cancer Biol.* 3 (6):391-398 (1992); Calabretta, B., et al., *Cancer Treatment Rev.* 19(2):169-179 (1993)), oncogenes/tumor suppressor genes (e.g., p53, Bayever, E., et al., *Antisense Research and Development* 3:383-390 (1993)), transcription factors (e.g., NF kappa B, Cogswell, P. C., et al., *J. Immunol.* 150(7):2794-804 (1993)) and viral genes (e.g., papillomaviruses, Cowser, L. M., et al., *Antimicrob. Agents and Chemo.* 37 (2):171-177 (1993); herpes simplex virus, Kulka, M., et al., *Antiviral Res.* 20(2):115-130 (1993)). To further illustrate, two RNA regions of the HIV-1 protein that can be targeted by the methods of the present invention are the REV-protein response element (RRE) and the TAT-protein transactivation response element (TAR). REV activity requires the presence of the REV response element (RRE), located in the HIV envelope gene (Malim, M. H., et al., *Nature* 338:254-257 (1989); Malim, M. H., et al., *Cell* 58:205-214 (1989)).

Further uses for anti-sense oligonucleotides are described in, e.g., U. S. Patent No. 5,599,922 to Gryaznov et al., incorporated herein by reference.

Methods of Administration

The tandem anti-sense oligonucleotides of the invention can be administered to a subject (including a warm-blooded animal, such as non-human mammals including dogs, cats, rats, mice, sheep, cattle, horses, pigs, as well as humans) to treat a disease state related to, or caused by, expression of mRNA (or other RNA or DNA, the transcription

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of which can be inhibited by anti-sense oligonucleotides). In a preferred embodiment, the disease state is characterized by a single point mutation. The tandem anti-sense oligonucleotides can be administered together or separately, and can be administered by any appropriate route of administration (e.g., oral, intravenous, transdermal, and the like). The antisense oligonucleotide molecules of the invention are typically administered to a subject or generated *in situ* (e.g., from a protected pro-drug form), such that they hybridize with or bind to cellular mRNA and/or genomic DNA. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. An example of a route of administration of an antisense nucleic acid molecule of the invention includes direct injection at a tissue site. Alternatively, an antisense nucleic acid molecule can be modified to target selected cells and then administered systemically. For example, for systemic administration, an antisense molecule can be modified such that it specifically binds to a receptor or an antigen expressed on a selected cell surface, e.g., by linking the antisense nucleic acid molecule to a peptide or an antibody which binds to a cell surface receptor or antigen. The antisense nucleic acid molecule can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

In yet another embodiment, the antisense nucleic acid molecule of the invention is an α -anomeric nucleic acid molecule. An α -anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gaultier et al. (1987) *Nucleic Acids Res.* 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-O-methylribonucleotide (Inoue et al. (1987) *Nucleic Acids Res.* 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue et al. (1987) *FEBS Lett.* 215:327-330).

30 *Pharmaceutical Compositions*

The antisense oligonucleotide molecules, (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject, e.g., a human. Such compositions typically comprise the anti-sense nucleic acid molecules and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with

pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, such media can be used in the compositions of the invention. Supplementary active compounds can also be
5 incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions
10 used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic
15 acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous
20 solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In
25 all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid
30 polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol,
35 ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be

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brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., two or more anti-sense oligonucleotides) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will
5 protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art.
10 The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

15 It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required
20 pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

The nucleic acid molecules of the invention can be inserted into vectors and used
25 as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see e.g., Chen et al. (1994) *PNAS* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene
30 delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or
35 dispenser together with instructions for administration.

Anti-sense Oligonucleotides

Tandem anti-sense oligonucleotides can be designed to be complementary to any desired target sequence. In preferred embodiments, at least one (or both) of the tandem anti-sense oligonucleotides is at least 5, 10, 15, 20, 30, 50, or 100 oligonucleotides in length. In certain preferred embodiments, at least one (or both) of the tandem anti-sense oligonucleotides is not longer than about 50, 40, 30, 25, 20, 15, 10, or 5 oligonucleotides in length.

It will be appreciated by those of ordinary skill in the art that additional tandem oligonucleotides can be provided in accordance with the invention. Thus, three, four, five or more tandem oligonucleotides, each binding to a portion or region of a target nucleic acid sequence substantially contiguous to at least one other region to which a tandem oligonucleotide binds. (See e.g., Maher et al. (1988) *Nucleic Acids Research* 16:3341-3358).

In preferred embodiments, the nucleic acids can be modified at the base moiety, sugar moiety or phosphate backbone to improve, e.g., the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acids can be modified to generate peptide nucleic acids (see Hyrup B. et al. (1996) *Bioorganic & Medicinal Chemistry* 4 (1): 5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, e.g., DNA mimics, which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup B. et al. (1996) *supra*; Perry-O'Keefe et al. PNAS 93: 14670-675.

PNAs can be used in therapeutic and diagnostic applications. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, e.g., inducing transcription or translation arrest or inhibiting replication. PNAs can also be used, e.g., in the analysis of single base pair mutations in a gene by, e.g., PNA directed PCR clamping; as 'artificial restriction enzymes when used in combination with other enzymes, e.g., S1 nucleases (Hyrup B. (1996) *supra*); or as probes or primers for DNA sequence and hybridization (Hyrup B. et al. (1996) *supra*; Perry-O'Keefe *supra*).

In another embodiment, PNAs can be modified, e.g., to enhance their stability or cellular uptake, by attaching lipophilic or other helper groups to PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques of drug delivery known in the art. For example, PNA-DNA chimeras can be generated which may

combine the advantageous properties of PNA and DNA. Such chimeras allow DNA recognition enzymes, e.g., RNase H and DNA polymerases, to interact with the DNA portion while the PNA portion would provide high binding affinity and specificity. PNA-DNA chimeras can be linked using linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup B. (1996) *supra*). The synthesis of PNA-DNA chimeras can be performed as described in Hyrup B. (1996) *supra* and Finn P.J. *et al.* (1996) *Nucleic Acids Research* 24 (17): 3357-63. For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry and modified nucleoside analogs, e.g., 5'-(4-methoxytrityl)amino-5'-deoxy-thymidine phosphoramidite, can be used as a between the PNA and the 5' end of DNA (Mag, M. *et al.* (1989) *Nucleic Acid Res.* 17: 5973-88). PNA monomers are then coupled in a stepwise manner to produce a chimeric molecule with a 5' PNA segment and a 3' DNA segment (Finn P.J. *et al.* (1996) *supra*). Alternatively, chimeric molecules can be synthesized with a 5' DNA segment and a 3' PNA segment (Peterser, K.H. *et al.* (1975) *Bioorganic Med. Chem. Lett.* 5: 1119-11124).

In other embodiments, the oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, e.g., Letsinger *et al.*, 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86:6553-6556; Lemaitre *et al.*, 1987, *Proc. Natl. Acad. Sci.* 84:648-652; PCT Publication No. W088/09810, published December 15, 1988) or the blood-brain barrier (see, e.g., PCT Publication No. W089/10134, published April 25, 1988). In addition, oligonucleotides can be modified with hybridization-triggered cleavage agents (See, e.g., Krol *et al.*, 1988, *BioTechniques* 6:958-976) or intercalating agents. (See, e.g., Zon, 1988, *Pharm. Res.* 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

Preparation of Antisense Oligonucleotides

An antisense oligonucleotide can be constructed using chemical synthesis procedures known in the art. An antisense oligonucleotide can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g. phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-

carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

Alternatively, an antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, nucleic acid transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest). Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the expression of the antisense RNA molecule in a cell of interest, for instance promoters and/or enhancers or other regulatory sequences can be chosen which direct constitutive, tissue specific or inducible expression of antisense RNA. The antisense expression vector is prepared as described above for recombinant expression vectors, except that the cDNA (or portion thereof) is cloned into the vector in the antisense orientation. The antisense expression vector can be in the form of, for example, a recombinant plasmid, phagemid or attenuated virus. The antisense expression vector is introduced into cells using a standard transfection technique, as described above for recombinant expression vectors.

Methods of Treatment

Another aspect of the invention pertains to methods for treating a subject, e.g., a human, having a disease or disorder characterized by (or associated with) the presence of a mutation in the genome, e.g., at least one gene or non-coding sequence of the subject. These methods include the step of administering at least two antisense oligonucleotides to the subject such that treatment occurs. Non-limiting examples of disorders or diseases characterized by or associated with mutations in the subject genome include proliferative disorders (e.g., cancers). Proliferative disorders are disorders which are associated with uncontrolled or undesirable cell proliferation. Examples of proliferative disorders include proliferative disorders of epithelial cells, e.g., proliferative disorders of gut derived cells, e.g., pancreatic cancer and colorectal cancer. These methods include administering to the subject an effective amount of at least two anti sense

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oligonucleotides such that treatment of the subject occurs. The terms "treating" or "treatment", as used herein, refer to reduction or alleviation of at least one adverse effect or symptom of a disease or disorder, e.g., a disease or disorder characterized by or associated with a genetic mutation. These modulatory methods can be performed *in vitro* (e.g., by culturing the cell with the agent) or, alternatively, *in vivo* (e.g., by administering the agent to a subject). In a preferred embodiment, the modulatory methods are performed *in vivo*, i.e., the cell is present within a subject, e.g., a mammal, e.g., a human, and the subject has a disorder or disease characterized by or associated with mutations.

10 A nucleic acid molecule used in the methods of treatment can be incorporated into an appropriate pharmaceutical composition described herein and administered to the subject through a route which allows the molecule, protein, modulator etc. to perform its intended function. Examples of routes of administration are also described *supra*.

15 The contents of all publications, patents and patent applications cited herein are hereby incorporated by reference.

Other equivalents are within the following claims.

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What is claimed is:

1. A method of treating a condition related to expression of mRNA, comprising administering to a subject in need thereof an effective amount of a first anti-sense oligonucleotide complementary to a first region of a target mRNA sequence and a
5 second anti-sense oligonucleotide complementary to a second region of the target mRNA sequence, the first and second anti-sense regions of the target mRNA sequence being substantially contiguous, such that the condition is treated.
- 10 2. The method of claim 1, wherein said first and second anti-sense oligonucleotides are each not longer than 30 bases.
3. The method of claim 1, wherein the target mRNA sequence is a proto-oncogene.
- 15 4. The method of claim 1, wherein the tandem anti-sense oligonucleotides form a duplex with the first and second anti-sense regions of the target mRNA, such that there is discrimination between the wild-type gene and the mutant gene of the subject.
5. The method of claim 1, wherein the subject is a human.
- 20 6. The method of claim 1, wherein the first anti-sense oligonucleotide is at least 5, 10, 15 and 20 oligonucleotides in length and the second anti-sense oligonucleotide is at least 25, 20, 15 and 10 respectively, so that when combined the final number of tandem, anti-sense oligonucleotides bound to the mutant gene totals not more than 30
25 oligonucleotide bases.
7. The method of claim 1, wherein the ratio of the length of the first anti-sense oligonucleotide to the length of the second anti-sense oligonucleotide is selected from the group consisting of at least 1:1, 1:2, 1:3, and 1:4.
- 30 8. A method of treating a subject suffering from a disorder characterized by the presence of a mutation in at least a portion of a gene of the subject, comprising administering to the subject in need thereof, an effective amount of at least two oligonucleotides preferentially binding to the site of the mutant gene such that the
35 subject is treated.

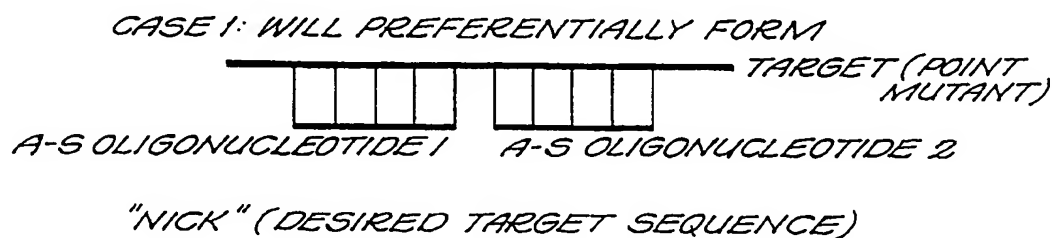
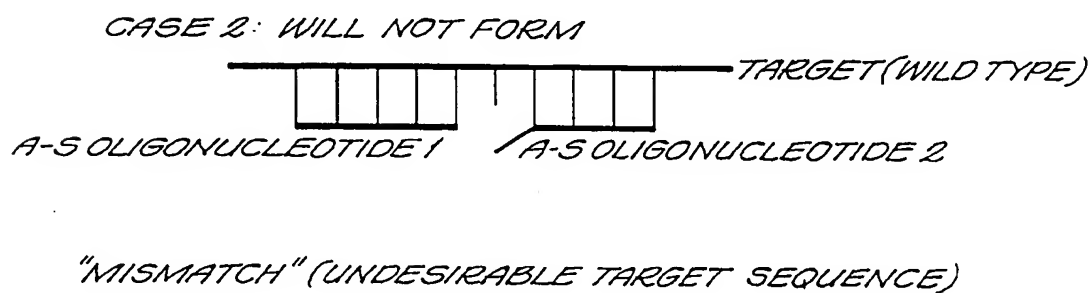
- 18 -

9. The method of claim 8, wherein the oligonucleotides are substantially contiguous.
10. The method of claim 8, wherein the oligonucleotides form a duplex which
5 discriminates between the wild-type gene and the mutant gene of the subject.
11. The method of claim 8, wherein the subject is a human.
12. The method of claim 8, wherein the first anti-sense oligonucleotide is at least 5,
10 10, 15 and 20 oligonucleotides in length and the second anti-sense oligonucleotide is at least 25, 20, 15 and 10 respectively, so that when combined the final number of tandem, anti-sense oligonucleotides bound to the mutant gene being not more than 30 oligonucleotide bases.
- 15 13. The method of claim 8, wherein the ratio of the length of the first anti-sense oligonucleotide to the length of the second anti-sense oligonucleotide is selected from the group consisting of at least 1:1, 1:2, 1:3, and 1:4.
14. A method of treating a subject suffering from a disorder characterized by the
20 presence of a mutation in at least a portion of a gene of the subject, comprising administering to the subject in need thereof in a pharmaceutically acceptable carrier, an effective amount of at least two oligonucleotides forming a duplex at the site of the mutant gene such that the subject is treated.
- 25 15. The method of claim 14, wherein the tandem anti-sense oligonucleotides when forming a duplex are substantially contiguous.
16. The method of claim 14, wherein the oligonucleotides form a duplex which
discriminates between the wild-type gene and the mutant gene of the subject.
- 30 17. The method of claim 14, wherein the subject is a human.
18. A method of treating a genetically transmitted hemolytic disease in a subject,
comprising administering to a subject in need thereof, an effective amount of the first
35 and second antisense oligonucleotides, forming a duplex and preventing gene expression, such that treatment occurs.

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19. The method of claim 18, wherein the disease is sickle cell anemia.

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***FIG. 1A******FIG. 1B***

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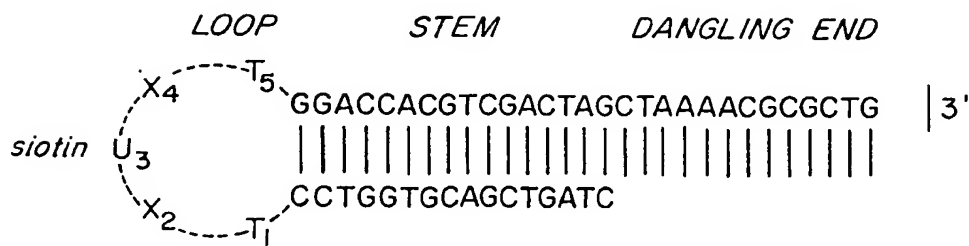
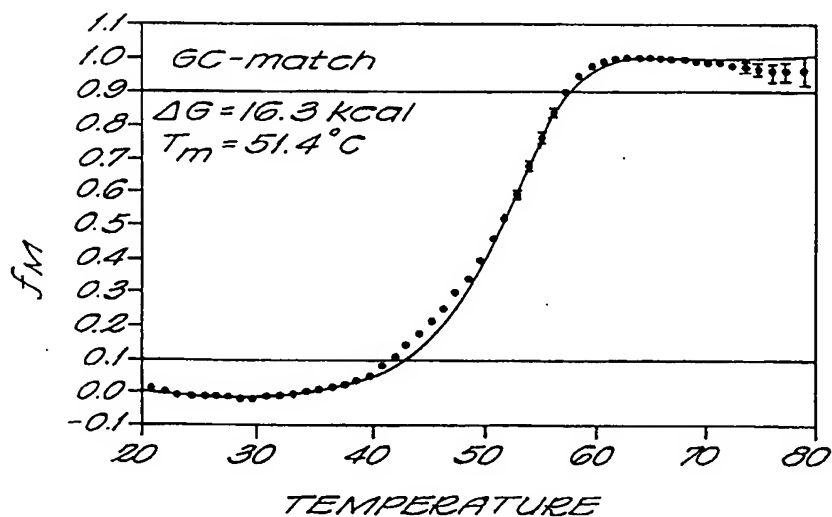
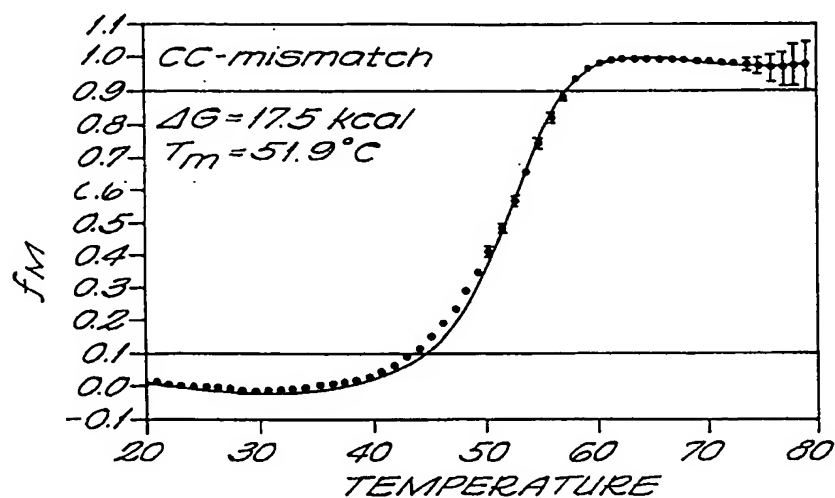
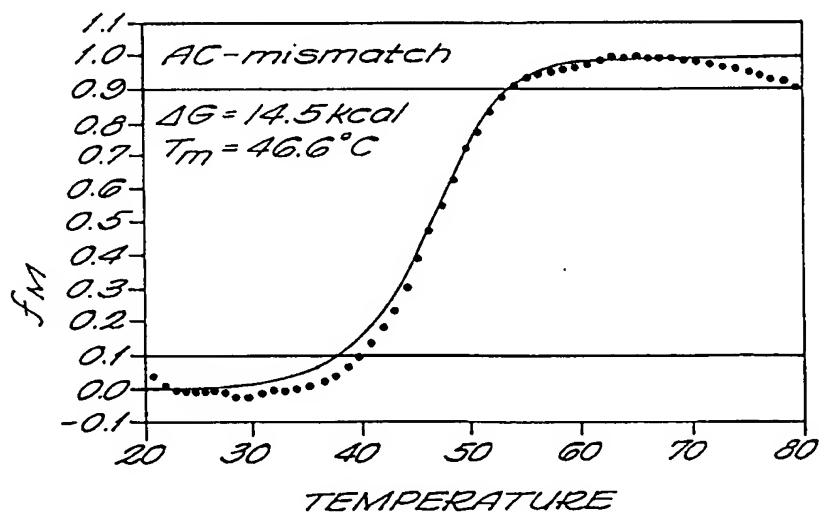
CASE

FIG. 2A	perfect nick formed	gattttgcgcgac
FIG. 2B	imperfect nick formed	cattttgcgcgac
FIG. 2C	imperfect nick formed	aattttgcgcgac
FIG. 2D	imperfect nick formed	tattttgcgcgac

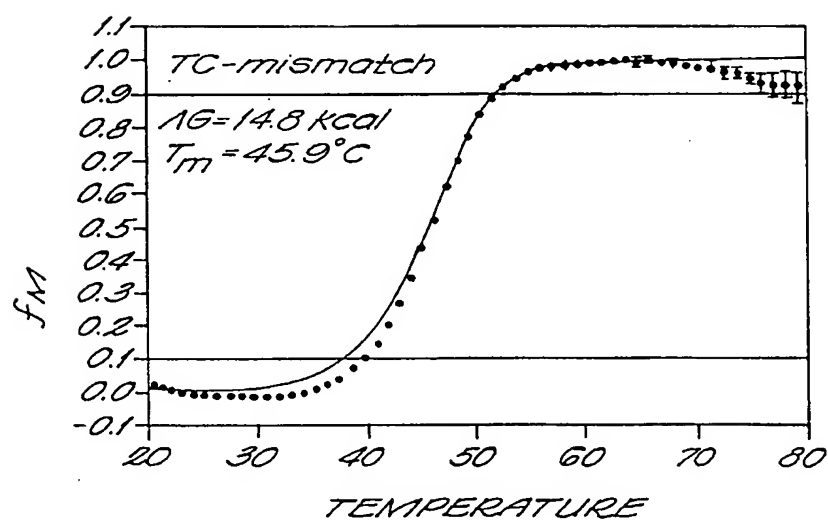
FIG. 2**FIG. 2A**

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**FIG. 2B****FIG. 2C**

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**FIG. 2D**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/03636

A. CLASSIFICATION OF SUBJECT MATTER IPC(6) : Please See Extra Sheet. US CL : Please See Extra Sheet. According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/ 6, 91.1, 91.31, 172.3, 320.1, 366; 536/ 23.1, 23/5, 24/31, 24/5; 514/44 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) DIALOG, BIOSIS, MEDLINE, DERWENT WPI, CA SEARCH, DERWENT BIOTECH ABS, US PAT.FULL. APS				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
Y	MAHER, L.J. et al. Comparative Hybrid Arrest by Tandem Antisense Oligodeoxyribonucleotides or Oligodeoxyribonucleoside Methylphosphonates In a Cell-Free System. Nucleic Acids Res. 1988. Vol. 16. No. 8. pages 3341-3357, see entire document.	1-19		
Y	GOODCHILD, J. Enhancement of Ribozyme Catalytic Activity by a Continuous Oligodeoxynucleotide (Facilitator) and by 2'-O-Methylation. Nucleic Acids Res. 1992. Vol 20. No. 17, pages 4607-4612, see entire document.	1-19		
Y	MERCOLA et al. Antisense Approaches to Cancer Gene Therapy. Gene Therapy. 1995. Vol 2, No. 1 pages 47-59, see entire document..	1, 3, 5, 8, 11, 14, 16-19		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.				
<table border="0"> <tr> <td> * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "B" earlier document published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed </td> <td> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "A" document member of the same patent family </td> </tr> </table>			* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "B" earlier document published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "A" document member of the same patent family
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "B" earlier document published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "A" document member of the same patent family			
Date of the actual completion of the international search 22 MAY 1998		Date of mailing of the international search report 18 JUN 1998		
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer MARK SHIBUYA Telephone No. (703) 308-0196		

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/03636

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DEAN, N. et al. Inhibition of Growth of Human Tumor Cell Lines in Nude Mice by an Antisense Oligonucleotide Inhibitor of Protein Kinase C-alpha Expression. Cancer Res. 01 August 1996. Vol 56. pages 3499-3507, especially pages 3505-3506.	1-19
Y	SCHWAB, G. Antisense Oligonucleotides Adsorbed to Polyalkylcyanoacrylate Nanoparticles Specifically Inhibit Mutated Ha-ras-Mediated Cell Proliferation and Tumorigenicity in Nude Mice. Proc Natl Acad Sci. October 1994. Vol 91. pages 10460-10464, especially pages 10461-10462.	1-19
Y	US 5,061,620 A (TSUKAMOTO et al.) 29 October 1991, column 7, lines 42-68.	18-19
Y	ROBBINS & COTRAN, Pathologic Basis of Disease, W.B. Saunders Company, Philadelphia. 1979. pages 723-726, especially 723.	18-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/03636

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C12Q 1/68; C12N 15/00, 15/64; C12P 19/34; C07H 21/00, 21/02, 21/04; A61K 48/00

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/ 6, 91.1, 91.31, 172.3, 320.1, 366; 536/ 23.1, 23/5, 24/31, 24/5; 514/44